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Microarray-based Functional Nanoproteomics for an Industrial Approach to Cancer. II Mass Spectrometry and Nanoconductimetry

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Abstract

Using the New England BioLabs (NEBL) SNAP-based Genes Expression in conjunction with our "sub-micron arrays" (Anodic Porous Allumina and/or Kapton based Nanopores), we exploit our proprietary microarrays scanner (DNASER, DNA analyzer) and Label Free Nanotechnologies to carry out the following tasks:

1) Construction of SNAP-based Genes Nanoarrays, using gold surface coated for 10 minutes with 2% solution of 3-Aminopropyltriethoxysilane (APTES) in acetone, rinsed in acetone and dried with filtered air. Full length complementary DNAs (cDNAs) for onco-suppressor 53 (p53), Cyclin-dependent kinase 2 (CDK2), SH2 (Src Homology 2) domain of the proto-oncogene tyrosine-protein kinase (Src) and tyrosine-protein phosphatase non-receptor type 11 (PTPN11) were amplified and cloned. Printing mix was prepared with 0.66 μg/μl DNA capture reagent BG-PEG-NH2 for the one-step synthesis of SNAP-tag substrates from esters on labels or surfaces;

2) Determination of Protein-Protein Interaction for the chosen cancer following the identification of leader genes (or hub genes, investigated with theoretical *ab initio* bioinformatics analysis using in-house software and algorithms, and then experimentally confirmed via DNASER). These genes are expressed by Pure (Protein synthesis Using Recombinant Elements) Express in spots less than 1 micron size piezo-microdispensed and then characterized via Label Free proprietary Autoflex Mass Spectrometry (MS) integrated with *ad hoc* software, namely the Spectrum Analyzer and Data Set manager (SpADS) and a proprietary Quartz Crystal Micro-balance with Dissipation factor monitoring (QCM_D) Nanoconductimetry, enabling to describe properties such as changes in frequency and conductance, viscoelasticity and dissipation factor. Solutions without DNA were prepared (called Master Mix, MM), as negative controls, in printing mix. Negative controls were prepared with a varying concentration range of SNAP capture reagent. As a positive control (for fluorescence analysis) mouse IgG or rabbit IgG (Pierce, IL, USA) were added in a printing mix instead of DNA.

Keywords

Quartz Crystal Microbalance with Dissipation Factor monitoring (QCM_D) and Nanoconductimetry, SNAP microarrays, Mass Spectrometry (MS)

Introduction

On the light of the sixteen recently published experimental papers, referenced in the last section [1-16], we have successfully completed the feasibility study on

two distinct but highly correlated lines of research in progress since 2012. On the basis of these obtained findings, we have subsequently prepared several proposals to be submitted as grant applications to the Small Business Innovation Research (SBIR) program, along with a small company based in the United States of America (USA), being presently identified.

We have identified the technologies we intend to use: namely, the New England BioLab (NEBL) proprietary "SNAP (Single Nucleotide Amplified Polymorphism) based Genes Expression" in conjunction with our proprietary "Sub-micron Arrays" (either our Anodic Porous Allumina – APA – Nanopores and/or Kapton based Nanopores, in cooperation with Arts Engineering), our proprietary microarrays scanner DNASER (DNA Analyzer) and Label Free Nanotechnologies resulting from a collaboration between the Laboratories of Biophysics and Nanobiotechnology (LBN) at Genoa University, Department of Experimental Medicine (DIMES), Genoa, Italy and Fondazione EL.B.A. Nicolini (FEN), Pradalunga (Bergamo), Italy.

Namely, we intend to carry out:

1) Construction of SNAP Genes Nanoarrays, using gold surface coated for 10 minutes with 2% solution of 3-Aminopropyltriethoxysilane – APTES – (Pierce, Rockford, IL, USA) in acetone, rinsed in acetone and dried with filtered air. Full length complementary DNAs (cDNAs) for onco-suppressor protein 53 (p53) and Cyclin-dependent kinase 2 (CDK2) were both purchased form DNASU Central Plasmid Repository located in the Biodesign Institute, Arizona State University (ASU), USA (https://dnasu.org/DNASU/Home.do). Full length cDNAs for the SH2 domain of the proto-oncogene tyrosine-protein kinase (Src) and tyrosine-protein phosphatase non-receptor type 11 (PTPN11) were purchased from Open Biosystem, Thermo Scientific. cDNAs were amplified and cloned into NdeI and XhoI sites in pCOATexp SNAPf vector, a derivative of pCOATexp and pSNAPf (further details can be found at (https://www.neb.com/products/n9183-psnapf-vector#tabselect0). Printing mix was prepared with 0.66 µg/µl DNA [bovine serum albumin, BSA, bound], capture reagent: BG-PEG-NH2 (an amine-terminated building block for the one-step synthesis of SNAP-tag substrates from NHS esters or other activated carboxyl esters on labels or surfaces, https://www.neb.com/products/s9150-bg-peg-nh2), ranging from 80 to 800 ng/µl [sBS3 bound].

2) Determination of Protein-Protein Interaction for the chosen cancer following the identification of genes leaders (hub genes, being highly interconnected and investigated via bioinformatics analysis and then experimentally confirmed via DNASER) expression by PureExpress in spots sized less than 1 micron size piezo-microdispensed (as well proprietary) and characterized via Label Free proprietary dedicated Autoflex Mass Spectrometry (MS) integrated with *ad hoc* software such as the proprietary Spectrum Analyzer and Data Set manager (SpADS) and a proprietary Quartz Crystal Micro-balance with Dissipation factor monitoring (QCM_D) Nanoconductimeter for performing nanoconductimetry. Solutions without DNA (master mix, or MM) were prepared, negative controls, in printing mix. Negative controls were prepared with a concentration range from 80 to 800 ng/µl of SNAP capture reagent. As a positive control (for fluorescence analysis) mouse IgG or rabbit IgG (Pierce, IL, USA) were added in a printing mix instead of DNA.

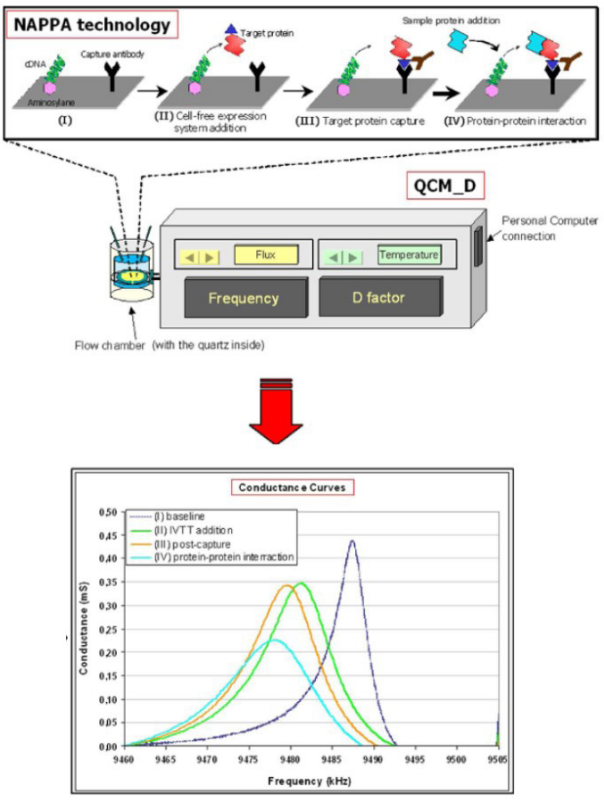

Figure 1: Our proprietary nanoconductomer for performing nanoconductimetry, developed by successfully coupling Quartz Crystal Micro-balance with Dissipation factor monitoring (QCM_D) with Nucleic Acid Programmable Protein Arrays (NAPPA) technology.

Results

In Figures 1-6 we show what we experimentally obtained. The results are obtained applying our innovative conductometer, realized by combining Nucleic Acid Programmable Protein Arrays (NAPPA) technology with QCM_D, to the characterization of protein-protein and protein-sterol interactions in a multiparametric way, taking advantage of the multiple information provided by the analysis of the conductance curves (i.e. conductance, viscoelasticity and adsorbed mass, which can be estimated using the Sauerbrey equation).

Moreover, through our Nanoconductometer we acquired information on the kinetic constant of enzymatic interaction. The protocol for properly performing Nanoconductimetric assay is shown in Figure 1. Piezoelectric inkjet printing offers the possibility of spotting on complex surfaces and using clinical samples of limited volume and amount, while QCM_D discriminates several genes per spot contrary to the single one with fluorescence [Figure 2]. We investigated well known pairs of interacting molecules like Cholesterol side-chain cleavage enzyme 11A1 (CYP11A1) and Cholesterol, Jun and Activating Transcription Factor 2 (ATF2), p53 and MDM2.

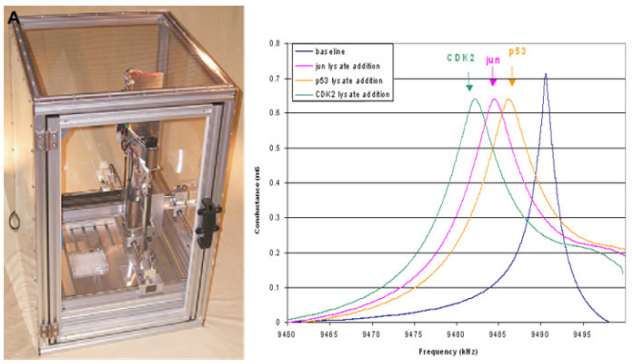

Figure 2: Piezoelectric liquid dispensing (left) for printing and probing of NanoProbeArrays of June, CDK2 and p53.

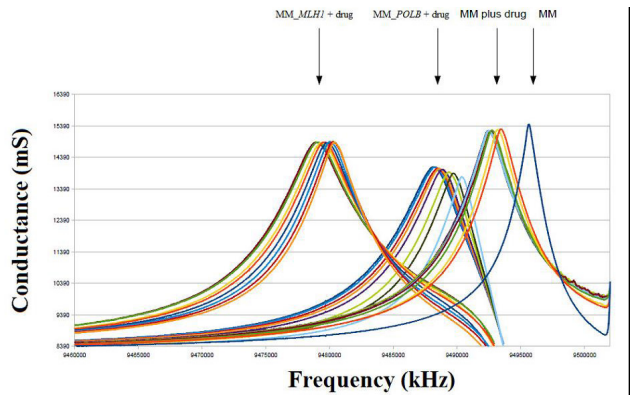

Figure 3: QCM_D conductance curves for MM_MLH1 and POLB interacting with Temozolomide at increasing concentrations.

An interesting implication for potential clinical applications concerned furthermore the possibility to drastically reduce the time of protein expression and capture under our experimental conditions.

The QCM_D instrument we used allowed us to monitor *in real time* the trend of Dissipation factor (D factor) and frequency (f) during the interaction between CYP11A1 and Cholesterol, both in solution and in blood. Assuming a Michaelis-Menten like behavior and fitting these experimental data, we were able to compute a constant (K) of about 100 μM, a value which is in good agreement with the values found in the extant literature. Finally, in order to verify the possibility to analyze simultaneously more interactions in a single NAPPA-Quartz Crystal (QC), we immobilized on the same QC up to three cDNAs. In our hands, we were able to successfully identify all of them and, subsequently, we analyzed the response to multi-protein interactions. Jun & CDK2 and Jun & CDK2 & p53 co-expressed in the same QCs were indeed tested for ATF2 interaction, both in flow and statically. Taken all together, we demonstrated the versatility of the NAPPA-QC biosensors for the detection of protein-protein interactions and protein-sterol interaction in cancer control as well as for controlling other diseases. Moreover, we measured also interaction between genes and their products and drugs (such as Temozolomide, commercially known as Temodar, an antiblastic used for brain cancer), showing that our proprietary instrument is able to perform both genomics and proteomics/ pharmacogenomics and pharmacoproteomics (shown in Figure 3). QCM_D is multi-purpose and, furthermore, is a very quick and effective tool for characterizing a biochemical system.

The analysis of self-assembling protein microarray NAPPA expressed with the SNAP tag in *Escherichia coli* coupled self free expression system prove capable to develop a highly standardized procedure in order to analyze the protein-protein interaction occurred on the array combining label free MS. We employ in the process PURE (Protein synthesis Using Recombinant Elements) system.

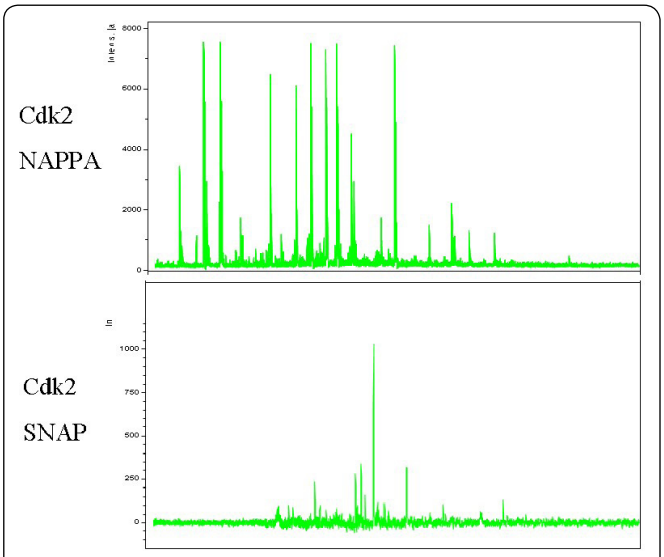

Figure 4: Mass Spectrometry NAPPA versus SNAP arrays.

For the first time an improved version of NAPPA, that allows for functional proteins to be synthesized *in situ* – with a SNAP tag – directly from printed cDNAs prepared just in time for the assay, has been expressed with a novel cell-free transcription/translation system reconstituted from the purified components necessary for *Escherichia coli* translation – the PURE system – and analyzed in a label free manner by three different mass spectrometry techniques, namely

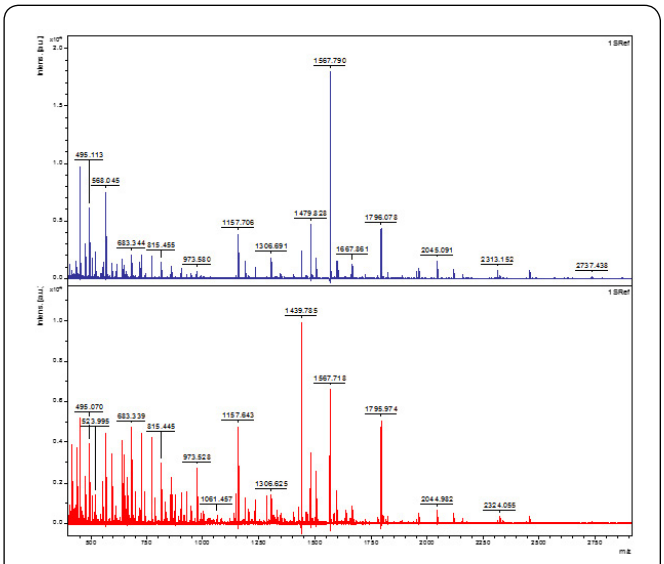

Figure 5: Mass Spectrometry of Snap-Nappa Arrays of p53 versus CdK2 genes expressed using PURE E. Coli system.

two Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF), a Voyager and a Bruker Ultraflex, and a Liquid Chromatography-Electrospray Ionization Mass Spectrometry (LC-ESIMS/ MS). Autoflex Mass Spectrometry shows discrimination of NAPPA versus SNAP arrays [Figure 4] and of different genes as p53 versus CdK2 genes expressed using PURE *E. Coli* system [Figure 5]. Due to the high complexity of the system, very difficult results were obtained and, therefore, in order to reduce this complexity and make sense of the findings, there was the need to develop and design an *ad hoc* bioinformatics tool for the analysis [Figure 6]. Furthermore, a contemporary fluorescence analysis of NAPPA, expressed by means of PURE system, has been performed to fully characterize this new NAPPA SNAP system and correlated to pure MS results.

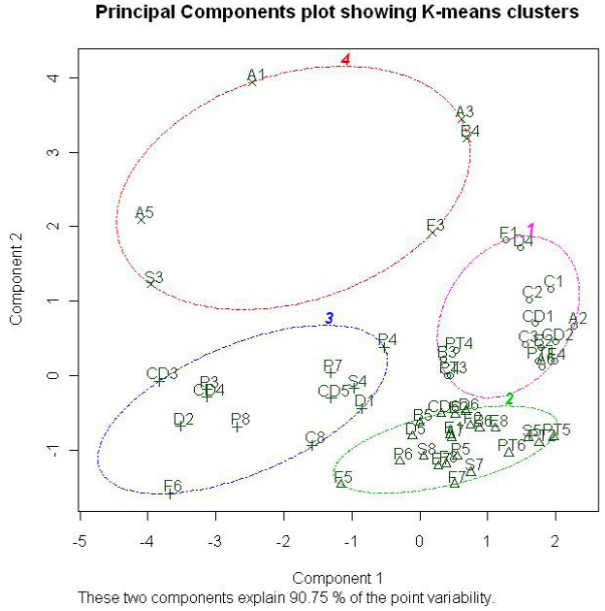

Figure 6: Cluster assignment for each known protein sample on a specimen of 56 spectra. Statistics are based on the SpADS results coupled with K Means clustering given in figure below. In bold assignment of clusters by human interpretation of cluster results. Highlited with * striking recognition.

We have recently developed a new MS bioinformatics procedure here reported for the first time. Instead of using a statistical approach, we started from all the components of the PURE mixture. All the hundred proteins of PureExpress mixture (15) have been indeed plotted against the peaks experimentally obtained of background acquired with our Mass Spectrometer [Figure 7].

The biophysical unit is m/z ratio (of PureExpress along x axis and of background experimentally acquired along y axis). All the peaks have been accordingly normalized, assuming constant equimolar ratios (1:1:1:.. for all the components), or, in other words, assuming that intensity of the peaks (relative abundance) is equal for all the components. Observing this scatterplot, an exceptional alignment of both components (peaks of PureExpress and those experimentally acquired) emerges. It can be anticipated from this scatterplot that, knowing the real, exact molar ratios, the signal would be more or less perfectly subtracted.

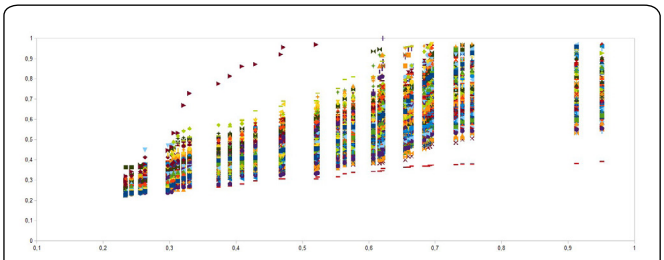

Figure 7: All the hundred proteins of PureExpress mixture of the E. coli system have been plotted against the peaks experimentally obtained of background acquired with our commercial Mass Spectrometer (AutoFlex, Bruker Daltonics). The biophysical unit is m/z ratio (of PureExpress along x axis and of background experimentally acquired along y axis). All the peaks have been accordingly normalized, assuming constant equimolar ratios.

Conclusion

The two combined Label Free technologies, MS integrated by proprietary software and technology, new *ad hoc* proprietary bioinformatics tools, and QCM_D Nanoconductometry integrated by proprietary hardware and software, as applied to optimized microarray with micron sized genes spots using SNAP and bacterial lysate technologies further optimized in a future application for SBIR program, may be constructed in such further optimized way to overcome multiple gene expression and interaction in a fashion unmatchable by current fluorescence technology using single gene, oversized spots. The approach described in Figure 7 is different from the clustering approach (shown in Figure 6) in that is more effective, less complex and can be routinely used also in clinical contexts.

Acknowledgments

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